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TEMPORAL EXPRESSION PATTERN OF CARBONIC ANHYDRASE GENE IN LOTUS JAPONICUS ROOT NODULE DEVELOPMENT AND DIFFERENT ORGANS

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Abstract: The relative abundance of cellular RNA transcripts is a commonly studied parameter of gene expression. Reverse transcription followed by the PCR (RT) - PCR leading to amplification of specific RNA sequence in cDNA form, is a sensitive means for detecting RNA molecules, a means for obtaining material for sequence determination and a step in cloning a cDNA copy of the RNA. The temporal expression pattern of carbonic anhydrase was determined in *Lotus japonicus* root nodule development and also in different organs.

INTRODUCTION

Carbonic anhydrase (CA) is a primitive and ubiquitous enzyme found in virtually every tissue and cell type, in many subcellular organelles and in organisms ranging from unicellular cyanobacteria through mammals (Badger and Price, 1994; Dodgson et al., 1991; Maren, 1967). The enzyme catalyzes the reverse hydration of CO_2 to bicarbonate. The widespread abundance of carbonic anhydrase isoforms in plants, animals and microorganisms suggests that possibly this enzyme participates in a broad range of diverge physiological and biochemical processes, including pH regulation, CO_2 and bicarbonate transport, ion transport and water end electrolyte balance (Henry, 1996). Metabolic roles include important steps in pyrimidine biosynthesis, gluconeogenesis and lipogenesis, as all these processes require bicarbonate for the initial carboxylation reactions. In green plants, the only well established physiological role of carbonic anhydrase is to provide adequate levels of inorganic carbon for carboxylases such as ribulose-biphosphate carboxylase (Rubisco). The physiological role of carbonic anhydrase in higher plants depends on its cellular localization. For plastid carbonic anhydrase, both the co-localization of Rubisco and carbonic anhydrase in the stroma and absence of carbonic anhydrase in non-green plants suggest a role in photosynthetic CO_2 fixation (Okabe et al., 1980).

The presence of carbonic anhydrase in non-green tissues such as roots, nodules and etiolated leaves suggests a function not necessarily coupled to photosynthesis, e.g. pH - buffering or facilitation of CO_2 transport across the plasma membrane (Graham et al., 1984). The presence of a non-photosynthetic related carbonic anhydrase activity in the plant nodules was first reported by Atkins in 1974. The role of CA in dark CO_2 fixation is also expected to be significant since it provides the substrate for the carboxylation of oxaloacetate by phosphoenolpyruvate carboxylase (Chollet et al., 1996). In symbiotic nitrogen fixation, dark CO_2 fixation may also play an important role. It has been suggested that the dark CO_2 fixation may provide a large fraction (30%) of the carbon skeletons for amide synthesis or bacteroid metabolism (Rosendal et al., 1990).

THE AIM OF INVESTIGATIONS

The aim of the study was to investigate the temporal expression of carbonic anhydrase gene in root nodule development and also, in different organs of *Lotus japonicus*.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Lotus japonicus (Cultivar Gifu B-129) seeds were kindly provided by Dr. Jens Stougaard (University of Aarhus, Denmark). The plants were grown in a controlled environment with a 18-h-day / 6h-night cycle, a 22°C day / 18°C night regime and 70% humidity (Handberg and Stougaard, 1992). Prior to germination, seeds were soaked for 5 min with H_2SO_4 and then sterilized for 20 min in a solution containing 2% NaOCl- 0.02% Tween 20. Seeds were pregerminated at 18°C in the dark for 72 h and the small plants were grown with Holland nutrient solution. For the inoculation with rhizobia, 72h seedlings were inoculated with a 0.1 OD600 suspension culture of *Mesorhizobium loti* (strain E1R.pMP2112) and the plants were grown in nitrogen-free BXD nutrient solution. The day of infection was considered day 0.

RT-PCR analysis

To analyze carbonic anhydrase gene expression, total RNA was isolated from different *Lotus japonicus* tissues (nodules, roots, leaves, stems, flowers, green seedpods, germinated cotyledons, germinated hypocotyls and apical meristem) and also from nodules in different stages of development (10, 14, 21 and 30 days post inoculation respectively), according to Brusslan and Tobin, 1992.

Prior to RT-PCR, the total RNA samples were treated with DNAse I (Promega, Madison, WI) at 37°C for 10 min, in order to eliminate any traces of contaminating genomic DNA.

For the reverse transcription and amplification of LjCA transcripts, Qiagen One Step RT-PCR system (Qiagen GmbH, Hilden, Germany) was used. For the gene in study, 1µg of total RNA (100ng) was reverse transcribed using the following designed primers: LjCA-beta type–F (5'-AGCTGAGGTACAGTGGAGTAGG-3')/LjCA-beta type–R (5'- TGAAGGTAGTGCCATGCTTCAG- 3') .

Reactions were run on a Gene Amp PCR system 9600 (Perkin Elmer) for 35 cycles of 95°C (15 min), 54°C (1 min) and 72°C (1 min).

The RT-PCR products were analyzed by 1.5% agarose gel electrophoresis, blotted on nylon membrane and hybridized to digoxigenin-11-rUTP-labelled inserts of LjCA beta type cDNA clone.

Membrane hybridization (16h) and washing (twice for 15 min in 2XSSC, 0.1% SDS and then 15 min in 0.1 XSSC, 0.1% SDS) were performed at 62°C according to standard protocols (Southern, 1975; Sambrook et al., 1989).

RESULTS AND DISCUSSIONS

To gain insight into the carbon metabolism in various tissues of Lotus *japonicus*, the accumulation of Lj CA beta-type transcripts was examined.

Plants used for the characterization of carbonic anhydrase expression were grown in Holland nutrient solution, (Flemetakis et al., 2002).

For this purpose, total RNA was isolated from different *Lotus japonicus* tissues (nodules, roots, leaves, stems, flowers, green seedpods, germinated cotyledons, germinated hypocotyls and apical meristem) and also from nodules in different stages of development (10, 14, 21 and 30 d.p.i. respectively). A semiquantitative reverse-transcription (RT)-PCR approach employed.

1µg of total RNA (100ng) treated with DNAse was used for each reaction, and the different RNA preparations were normalized by parallel amplification of the constitutively expressed gene Lj Ubiquitin using LjUBQ-F(5'–ATGCAGATCTTTTGTGAAGAC-3') and Lj UBQ–R (5'-

ACCACCACGGAAGACGGAG- 3') primers. Under our experimental conditions, an exponential increase of the amplification products was observed until after 35

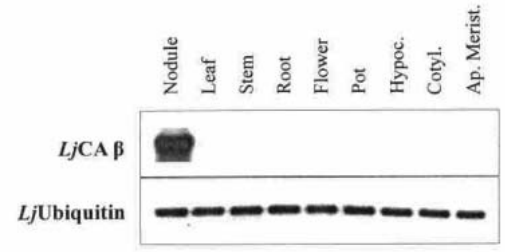

Figure 1. Accumulation of Lj CA mRNAs in Lotus japonicus non-symbiotic tissues.

amplification cycles. All the amplification reaction was performed under these conditions in order to obtain semi-quantitative results.

Accumulation of Lj CA mRNA was detected only in nodules, where the abundance of transcript was considerably high. This result might suggest that LjCA is a true nodulin gene.

In all the other tested tissues, no hybridization signal was observed. (Figure 1). Total RNA was isolated from various tissues as indicated and subjected to semi-quantitative RT-PCR analysis using L. japonicus ubiquitin as an internal control.

The accumulation of Lj CA beta-type transcript was also examined after the infection 3 days old plantlets with Mesorhizobium loti (strain E1R.pMP2112) using semi-quantitative reverse-transcription (RT)-PCR. It should be pointed out that nodules were visible at 10 d.p.i., whereas in earlier stages, the inoculated region of the root was examined. The accumulation of Lj CA beta-type transcript was first detectable in infected root segments as 5 d.p.i., preceding the induction of nitrogenase, reached a maximum at 14 d.p.i. and then gradually decreased (Figure 2).

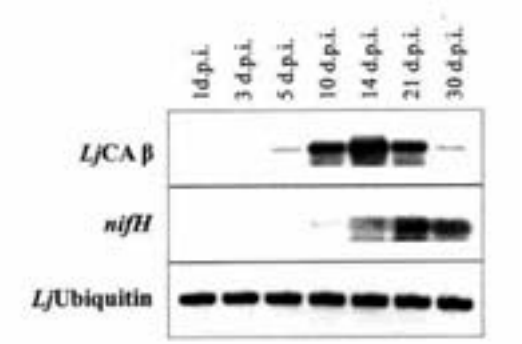

Figure 2. Accumulation of Lj CA beta type mRNAs in L. japonicus root nodule development. The expression levels at infected roots 1 and 3d.p.i. with Mesorhizobium loti and root nodules at 10, 14, 21 and 30 d.p.i.

As a positive control and tool to asses nitrogenase activity during nodule development, M. loti nifH gene transcripts encoding nitrogenase reductase were amplified MlnifH-F (5 –AGGATACGGTTCTGCATC-3) and MlnifH-R (5– GCATACTGGATTACCGTC- 3) primers. The expression of nifH was first detected at low levels 10 days post-infection and then reached a high constant level throughout nodule development.

CONCLUSIONS

The data showed that LjCA beta type transcripts appeared to accumulate at high levels only in nodules.

In all the other tissues tested, no hybridization signal was observed.

The level of carbonic anhydrase expression varies with the stage of nodule development.

In young nodules (14 days post-infection), expression of carbonic anhydrase exhibited maximum levels of accumulation.

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